

DESCRIPTION

NOVEL FUSION PROTEIN, GENE THEREOF,
RECOMBINANT VECTOR BEARING THE GENE
AND RECOMBINANT VIRUS AS WELL AS USE THEREOF

TECHNICAL FIELD

The present invention relates to a novel fusion polypeptide of a polypeptide having the antigenicity of Mycoplasma gallisepticum and a polypeptide derived from the outer membrane protein of herpes viruses, a hybrid DNA coding for the fusion polypeptide, and a recombinant Avipox virus bearing the hybrid DNA, as well as a vaccine using the recombinant Avipox virus.

BACKGROUND ART

Mycoplasma gallisepticum (hereinafter sometimes abbreviated as MG) is a bacterium that causes reduction in an egg-laying rate and a hatching rate of eggs for poultry including chicken. This causative MG is widely spread all over the world so that a great deal of damage has been done to the poultry farming. For the prevention of MG, an inactivated vaccine or a live vaccine is currently utilized. However, the former live vaccine involves disadvantages of complicated inoculation procedures, short duration of immunity, expensive etc. The latter vaccine has such a defect that an unexpected

disease might be developed by use in combination with live vaccine for other disease. Another disadvantage is that MG agglutination reaction system, which makes rapid detection of MG infection possible, can not be used for
 5 both inactivated and live vaccines.

It is expected that a protein derived from MG such as its antigenic protein for preventing from MG infection would be produced by genetic engineering technology and utilized as a vaccine.

10 The production system of the antigenic protein of Mycoplasma gallisepticum using E. coli or yeast by means of genetic engineering (JPA 2-111795, etc.) encounters such problems that depending upon a protein to be expressed, the antigenic protein is only expressed in
 15 a less amount, proteins of host origin might be by-produced and intermingled, host-derived pyrogen is removed only with difficulty, etc. For these reasons, studies are still focused on a recombinant virus to prepare antigenic proteins or on a recombinant live
 20 vaccine.

The expression of foreign genes using recombinant viruses, in most cases, genes of eucaryotes or viral genes are expressed. For this reason, addition or expression mode of sugar chains or the like is similar
 25 to the protein expression mechanism in infected cells. Thus, induction of an antibody titer to the expressed protein was relatively easy in vivo. However, genes of prokaryotes are rarely expressed in recombinant viruses.

Because of different expression mode between eukaryotes and prokaryotes, it was difficult to say that a specific antibody was effectively induced (Austen et al., Protein Targeting and Selection, Oxford Univ. Press (1991)).

5 Turning to MG, recombinant viruses in which a gene coding for the protein has been incorporated are known by JPA 5-824646 and JPA 7-133295, WO 94/23019, etc. In particular, WO 94/23019 reveals that when a recombinant virus capable of expressing the antigenic
10 protein of MG having a viral membrane anchoring region, which is obtained by ligating the signal membrane anchoring portion of HN gene of New Castle disease virus (hereinafter abbreviated as NDV) with the antigenic gene of MG, is inoculated as a recombinant live vaccine, the
15 antibody is induced more effectively than a recombinant virus capable of expressing the antigenic gene of MG alone.

 However, expression to such an extent is not always sufficient to achieve the desired effect as a
20 vaccine.

 Therefore, it is the urgent need to find an improved method for higher recognition of the antigen in order to develop an effective vaccine against MG infections.

25 Outer membrane proteins other than NDV mentioned above are known also in the genus Herpesvirus, etc. With respect to glycoproteins B(gB), C(gC), D(gD), H(gH) and I(gI) of herpes simplex viruses; proteins gBh,

gCh, gDh, gHh and gIh of Marek's disease viruses (hereinafter often referred to as MDV) corresponding to herpes simplex virus glycoproteins gB, gC, gD, gH and gI and proteins of the genus Herpesvirus homologous to those proteins described above, etc., the nucleotide sequence and amino acid sequence of these proteins are known. It is also known that a part of these proteins induces neutralizing antibodies of herpes simplex viruses (Deluca et al., Virology, 122, 411-423 (1982)). It is further known that neutralizing antibodies can be induced by incorporating genes coding for these proteins into vaccinia viruses and expressing the genes (Blacklaws et al., Virology, 177, 727-736 (1990)).

However, investigations to make use of signal sequences of such outer membrane proteins of the genus Herpesvirus were hardly made so far.

DISCLOSURE OF THE INVENTION

Under the situation of the prior art stated above, the present inventors have made extensive studies to provide a recombinant virus capable of expressing a Mycoplasma antigenic protein having an enhanced infection prevention activity in large quantities, which allows a host to recognize the antigen highly efficiently. As a result, it has been found that by infecting to a host a recombinant Avipox virus, in which a hybrid DNA obtained by ligating a DNA of the outer membrane protein of the genus Herpesvirus with a DNA of the antigenic protein of

Mycoplasma has been inserted, the antigen recognizing ability of the host can be markedly improved. The present invention has thus been accomplished.

Accordingly, the present invention provides:

- 5 a fusion protein comprising a polypeptide having the antigenicity of Mycoplasma gallisepticum (hereinafter sometimes referred to as Mycoplasma-derived polypeptide) and a polypeptide derived from the outer membrane protein of a herpes virus (hereinafter sometimes referred to as Herpesvirus-derived polypeptide)
- 10 characterized in that the polypeptide derived from outer membrane protein is ligated with the polypeptide having the antigenicity of Mycoplasma gallisepticum at the N terminus thereof;
- 15 a hybrid DNA coding for the fusion protein;
a recombinant Avipox virus in which the hybrid DNA has been incorporated; and,
a live vaccine comprising the recombinant Avipox virus as an effective ingredient.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a drawing for explaining procedures for construction of pNZ40K-S.

Fig. 2 is a drawing for explaining procedures for construction of pNZ40K-S.

25 Fig. 3 is a drawing for explaining procedures for construction of pNZ40K-S.

Fig. 4 is a drawing for explaining procedures

for construction of pNZ40K-C.

Fig. 5 is a drawing for explaining procedures for construction of pNZ40K-C.

Fig. 6 is a drawing for explaining procedures
5 for construction of pNZ40K-C.

Fig. 7 shows the results of Western blotting by which expression of TTM-1 polypeptide was confirmed.

Fig. 8 shows scores of the tracheal lesion caused.

10 BEST MODE FOR PRACTICING THE INVENTION

Mycoplasma-derived polypeptides and genes therefor

In the present invention, the term Mycoplasma-derived polypeptides is used to mean the antigenic proteins that cause an antigen-antibody reaction with MG
15 immune serum or MG infected serum and that are derived from MG. These polypeptides are not restricted to proteins per se that native Mycoplasma gallisepticum expresses, and may include modified polypeptides. For example, one or more amino acids of the polypeptides may
20 be modified naturally or artificially in a conventional manner such as site-specific mutation, etc. (JPB 6-16709, etc.) through loss, addition, insertion, deletion, substitution, etc. Of course, the proteins, even after such modification, should contain the epitope showing the
25 antigenicity. For determination of the epitope region, there are available known methods based on the peptide scanning technique such as the method of Geysen et al.

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(J. Immunol. Meth., 102, 259-274 (1987)), the method of Hopp et al., (Proc. Natl. Acad. USA, 78, 3824-3828 (1981)), the method Chou et al. (Advances in Enzymology, 47, 145-148 (1987)), etc.

5 Specific examples of the peptides having the antigenicity include antigenic proteins disclosed in JPA 2-111795 (U.S. Patent Application Serial Nos. 359,779, 07/888,320 and 08/299,662), JPA 5-824646 (U.S. Patent No. 5,489,430), WO 94/23019 (U.S. Patent Application Serial
10 No. 08/525,742, JPA 6-521927) and proteins of Mycoplasma gallisepticum containing the amino acid sequences of those proteins. Of course, so long as the epitope is contained therein, a part of the peptides described above may also be usable.

15 Of these peptides, preferred are the polypeptide of about 40 kilodaltons (kd) described in JPA 5-824646, the polypeptide of about 66 kd encoded by TM-66 gene and the polypeptide of about 67 kd encoded by TM-67 gene described in JPA 5-521927, which are
20 designated as SEQ NO: 16 and SEQ NO: 27 therein.

 In the present invention, genes of the Mycoplasma-derived polypeptides bear DNA sequences coding for the polypeptide having the antigenicity of Mycoplasma gallisepticum described above. Such DNA can be obtained
25 by synthesis or acquired from wild bacteria belonging to Mycoplasma gallisepticum. Specific examples of such bacteria are strains R, S6, KP-13, PG31, etc. DNA may also be derived from MG isolated from wild strains.

These genes can also be modified by loss, addition, insertion, deletion, substitution, etc. in a conventional manner as described in Methods in Enzymology, etc.

Herpesvirus-derived polypeptides and genes thereof

5 The Herpesvirus-derived polypeptides in the present invention refer to polypeptides derived from proteins that construct an envelope of viruses belonging to the genus Herpesvirus. The Herpesvirus-derived polypeptides may not always be the full length of the
10 proteins. Where the polypeptides are used solely to be expressed on the surface of cell membranes function as fusion proteins, it is sufficient for the polypeptide to contain a membrane anchor and a signal sequence therein, and where the polypeptides are employed for secretion,
15 the polypeptides may contain only a signal sequence for that purpose. The outer membrane proteins may be either type I or type II of the outer membrane proteins. The signal sequence and the membrane anchoring sequence are both readily detectable by analyzing the amino acid
20 sequence in the hydrophobic peptide region at the carboxyl terminus or amino terminus thereof.

 Specific examples of the outer membrane protein include gB, gC, gD, gH and gI which are glycoproteins of herpes simplex viruses, and gBh, gCh, gDh, gHh and gIh of
25 MDV corresponding to herpes simplex viruses glycoproteins gB, gC, gD, gH and gI, and proteins of the genus Herpesvirus homologous to the proteins described above.

Of course, polypeptides bearing the epitope other than the signal sequence of the outer membrane proteins may also be ligated with the aforesaid polypeptides having the antigenicity. By the ligation it
5 is expected that the epitope will give the immunity to the living body in vivo.

In the present invention, the genes for the Mycoplasma-derived polypeptides contain DNA sequences coding for the Herpesvirus-derived polypeptides described
10 above and such DNAs can be synthesized or acquired from naturally occurring herpes viruses. These genes may also be modified by loss, addition, insertion, deletion, substitution, etc. in a conventional manner as described in Methods in Enzymology, etc.

15 Fusion protein and hybrid DNA

The fusion proteins of the present invention are obtained by incubating a recombinant Avipox virus inserted hybrid DNA, which will be later described, in culture cells such as chick embryo fibroblast cells
20 (hereinafter referred to as CEF cells) or embryonated chorioallantoic membrane cells, etc.

The thus obtained fusion proteins can be employed as a component vaccine.

The hybrid DNA of the present invention
25 comprises the gene for the Mycoplasma-derived polypeptide and the gene for the Herpesvirus-derived polypeptide, which are ligated with each other directly or via an

optional DNA sequence.

The hybrid DNA of the present invention can be produced in a conventional manner, for example, by a method in which the outer membrane protein and the antigenic protein of Mycoplasma gallisepticum are digested with restriction enzymes, respectively, and the resulting ligatable DNA fragment coding for the outer membrane protein of herpes viruses or for the signal sequence of the outer membrane protein is ligated with the resulting ligatable DNA fragment coding for the antigenic protein of Mycoplasma gallisepticum, using a ligase directly or via an appropriate linker.

Specific examples of the amino acid sequences for the fusion proteins of the present invention include SEQ^{ID} NO: 2 and SEQ^{ID} NO: 4. The sequence of the antigenic protein of 40 kilodaltons derived from Mycoplasma gallisepticum is found in amino acids 64-456 of SEQ NO: 2 and in amino acids 693-1086 of SEQ^{ID} NO: 4. The signal sequence of outer membrane protein gB derived from MDV is found in amino acids 1-63 of SEQ^{ID} NO: 2. In SEQ^{ID} NO: 4, amino acids 1-672 correspond to almost the full length of outer membrane protein gB derived from MDV. Specific examples of nucleotide sequences of the hybrid DNAs coding for these fusion proteins are those shown by SEQ^{ID} NO: 1 and SEQ^{ID} NO: 3.

These fusion proteins and hybrid DNAs are given by way of examples but are not deemed to be limited thereto.

Recombinant Avipox virus

The recombinant Avipox virus of the present invention is a recombinant Avipox virus in which the aforesaid DNA or hybrid DNA has been inserted in the non-essential region. The recombinant Avipox virus of the present invention can be constructed in a conventional manner, e.g., by the method described in Japanese Patent Application Laid-Open No. 1-168279. That is, the non-essential region of Avipox virus is incorporated into a DNA fragment to construct a first recombinant vector.

As the non-essential region of Avipox virus which is used in the present invention, there are a TK gene region of quail pox virus, a TK region of turkey pox virus and DNA fragments described in JPA 1-168279, preferably a region which causes homologous recombination with EcoRI fragment of about 7.3 Kb, HindIII fragment of about 5.2 Kb, EcoRI-HindIII fragment of about 5.0 Kb, BamHI fragment of about 4.0 Kb, described in the patent specification supra.

Examples of the vector used in the present invention include plasmids such as pBR322, pBR325, pBR327, pBR328, pUC7, pUC8, pUC9, pUC18, pUC19, and the like; phages such as λ phage, M13 phage, etc.; cosmid such as pHc79 and the like.

The Avipox virus used in the present invention is not particularly limited so long as it is a virus infected to avian. Specific examples of such a virus include pigeon pox virus, fowl pox virus (hereafter

abbreviated as FPV), canary pox virus, turkey pox virus, preferably pigeon pox virus, FPV and turkey pox virus, more preferably pigeon pox virus and FPV. Specific examples of the most preferred Avipox virus include FPVs
5 such as ATCC VR-251, ATCC VR-249, ATCC VR-250, ATCC VR-229, ATCC VR-288, Nishigahara strain, Shisui strain, CEVA strain and a viral strain among CEVA strain-derived viruses which forms a large plaque when infected to chick embryo fibroblast, and a virus such as NP strain (chick
10 embryo-attenuated pigeon pox virus Nakano strain), etc. which is akin to FPV and used as a fowlpox live vaccine strain. These strains are commercially available and readily accessible.

Next, the hybrid DNA of the present invention
15 is inserted into the non-essential region of the first recombinant vector described above to construct a second recombinant vector. In general, the hybrid DNA employed may have any nucleotide sequence, irrespective of synthetic or natural one, so long as the hybrid DNA
20 effectively functions as a promoter in the system of transcription possessed by Avipox viruses. Accordingly, not only promoters inherent to Avipox viruses such as promoters for Avipox virus-derived genes coding for thymidine kinase but also DNAs derived from viruses other
25 than Avipox viruses and DNAs derived from eukaryotes or prokaryotes may also be employed in the present invention, insofar as these substances meet the requirements described above. Specific examples of such

promoters include promoters for vaccinia viruses (hereinafter often referred to as VV) as described in Journal of Virology, 51, 662-669 (1984), more specifically, a promoter of VV gene coding for 7.5 K polypeptide, a promoter of VV gene coding for 19 K polypeptide, a promoter of VV gene coding for 42 K polypeptide, a promoter of VV gene coding for thymidine kinase, a promoter of VV gene coding for 28 K polypeptide, etc. Furthermore, there may be used a synthetic promoter obtained by modification of the Moss et al. method (J. Mol. Biol., 210, 49-76 and 771-784, 1989), Davidson's synthetic promoter, a promoter obtained by modifying a part of the Davidson's promoter through deletion or change in such a range that the promoter activity is not lost (e.g.,

TTTTTTTTTTTTTGGCATATAAATAATAAATACAATAATTAATTACGCGTAAAAA
 a TTGAAAAACTATTCTAATTTATTGCACTC, (Seq ID NO:5)
 TTTTTTTTTTTTTTTTTTTTTTGGCATATAAATAATAAATACAATAATTAATTACGCGT
 a AAAAATTGAAAAACTATTCTAATTTATTGCACTC etc.). (Seq ID NO:6)

20 Further in view of easy detection of the recombinant virus, a marker gene such as a DNA coding for β -galactosidase may also be inserted.

The recombinant Avipox virus can be constructed by transfecting the second recombinant vector described above to animal culture cells, which has been previously
 25 infected with Avipox virus, and causing homologous recombination between the vector DNA and the viral genome DNA. The animal culture cells used herein can be any

cells, so long as Avipox can grow in the cells. Specific examples of such animal culture cells are CEF cells, embryonated egg chorioallantoic membrane cells, and the like.

- 5 The objective recombinant Avipox virus is isolated from the virus infected to host cells by plaque hybridization, etc.

Live vaccine

- The recombinant virus of the present invention
10 constructed by the method described above can be inoculated to avian as a live vaccine for Mycoplasma gallisepticum infection.

- The live vaccine of the present invention is prepared by, e.g., the following method, though the
15 process is not particularly limited thereto. The recombinant virus of the present invention is infected to cells in which the virus can grow (hereafter referred to as host cells). After the recombinant virus grows, the cells are recovered and homogenated. The homogenate is
20 centrifuged to separate into the precipitates and the high titer supernatant containing the recombinant virus. The resulting supernatant is substantially free of host cells but contains the cell culture medium and the recombinant virus and hence can be used as a live
25 vaccine. The supernatant may be diluted by adding a pharmacologically inert carrier, e.g., physiological saline, etc. The supernatant may be freeze-dried to be provided for use as a live vaccine. A method for

administration of the live vaccine of the present invention to fowl is not particularly limited and examples of the administration include a method for scratching the skin and inoculating the live vaccine on the scratch, effecting the inoculation through injection, oral administration by mixing the live vaccine with feed or drinking water, inhalation by aerosol or spray, etc. In order to use as the live vaccine, the dosage may be the same as ordinary live vaccine; for example, approximately 10^2 to 10^8 plaque forming unit (hereinafter abbreviated as PFU) is inoculated per chick. Where the inoculation is effected by injection, the recombinant virus of the present invention is generally suspended in about 0.1 ml of an isotonic solvent such as physiological saline and the resulting suspension is provided for use. The live vaccine of the present invention can be lyophilized under ordinary conditions and can be stored at room temperature. It is also possible to freeze the virus suspension at -20 to -70°C and store the frozen suspension.

Particularly where the genes coding for the polypeptides derived from the outer membrane proteins of herpes viruses described above are those coding for polypeptides having more than one epitope of herpes viruses, preferably having at least 90% homology to native outer membrane proteins, the live vaccine of the present invention functions as a vaccine for both Mycoplasma gallisepticum infection and Avipox viral

infection. In addition, the live vaccine of the present invention can also function as an effective vaccine for infection with herpes virus originating from outer membrane proteins. That is, the live vaccine of the present invention can be used as a so-called trivalent vaccine.

EXAMPLES

Example 1

Construction of recombinant pNZ40K-S bearing hybrid DNA ligating TTM-1 protein DNA immediately after the signal of gB gene for Marek's disease virus (cf. Figs. 1, 2 and 3)

First, plasmid pUCgB bearing gB gene of Marek's disease virus, disclosed in JPA 6-78764, was digested with restriction enzymes BamHI and SalI to recover a fragment of 3.9 kb.

Separately, plasmid pGTPs was constructed by digesting plasmid pNZ1729R (Yanagida et al., J. Virol., 66, 1402-1408 (1992)) with HindIII and SalI, inserting the resulting DNA fragment of about 140 bp into pUC18 at the HindIII-SalI site thereof, further inserting synthetic DNA (5'-AGCTGCCCCCCCCGCAAGCTTGCA-3')^(SEQ ID NO:7) at the HindIII-PstI site, then inserting synthetic DNA (5'-TCGACATTTTATGTGTAC-3')^(SEQ ID NO:8) at the SalI-EcoRI site and finally inserting synthetic DNA (5'-AATCGGCCGGGGGGGCCAGCT-3')^(SEQ ID NO:9) at the SacI-EcoRI site.

The thus obtained pGTPs was digested with

restriction enzymes SalI and BamHI and then ligated with the aforesaid 3.9 kb fragment using a ligase to obtain pGTPsMDgB. Thereafter, pNZ2929XM1 disclosed in WO 94/23019 was digested with EcoRI to recover a fragment of 740 bp and then obtained a blunt end with T4 DNA polymerase. On the other hand, pGTPsMDgB was also digested with XbaI and then obtained a blunt end with T4 DNA polymerase. Subsequently, pGTPsMDgB was ligated with the 740 bp fragment having the blunt end using a ligase to construct a new plasmid. This new plasmid was digested with BglII and SalI to recover a fragment of 3.0 kb. The 3.0 kb fragment was ligated with the 1.1 kb fragment obtained through digestion of pNZ2927XM1 with BglII and SalI, using a ligase. Thus, there was obtained a plasmid ligating the N terminus of TTM-1 gene at the C terminus of the signal sequence of gB gene of Marek's disease virus.

Finally, a fragment of 1.4 kb obtained by digestion of pGTPs40K-S with SalI and BamHI was ligated with a fragment of 9.3 kb obtained by digestion of plasmid pNZ1829R with SalII and BamHI, using a ligase. The objective plasmid pNZ40K-S of 10.7 kb was thus constructed for use in recombination.

Example 2

Construction of recombinant pNZ40K-C bearing hybrid DNA ligating TTM-1 protein DNA at the C terminus of gB gene for Marek's disease virus (cf. Figs. 4, 5 and 6)

After plasmid pGTPsMDgB obtained in Example 1 was digested with restriction enzyme MluI, and then obtained a blunt end with T4 DNA polymerase, which was followed by digestion with restriction enzyme XbaI to recover a fragment of 1.9 kb. Separately, pBluescriptII (made by Toyobo Co., Ltd., hereinafter abbreviated as pBSKSII) was digested with restriction enzymes XbaI and SmaI. The resulting fragment was ligated with the 1.9 kb fragment obtained above using a ligase to give a plasmid.

The resulting plasmid was digested with restriction enzymes EcoRI and SalI. The resulting fragment was ligated with the 550 bp fragment and the 615 bp fragment, both obtained by digestion of pNZ2929XM1 with restriction enzymes EcoRI and SalI and with restriction enzymes EcoT22I and SalI, respectively, using a ligase to construct a plasmid. The thus obtained plasmid was digested with restriction enzymes XbaI and SalI. The resulting 2.7 kb fragment was ligated with the 3.3 kb fragment obtained by digestion of pGTPsMDgB with restriction enzymes XbaI and SalI, using a ligase.

Plasmid pGTPs40K-C ligating the TTM-1 gene at the N terminus thereof with the gB gene for Marek's disease virus at the C terminus thereof was thus obtained.

Finally, a fragment of 2.7 kb obtained by digestion of pGTPs40K-C with SalI and XbaI was ligated with a fragment of 9.5 kb obtained by digestion of plasmid pNZ1829R with SalI and XbaI, using a ligase. The objective plasmid pNZ40K-C of 12.2 kb for recombination

was thus constructed.

Example 3

Construction of recombinants FPV 40K-C and 40K-S and purification thereof

- 5 NP strain, which is a fowlpox live vaccine strain, was infected to monolayered CEF at m.o.i. = 0.1. Three hours after, these cells were scraped off from the monolayer by a treatment with trypsin to form a cell suspension. After 2×10^7 cells in the suspension were
 10 mixed with 10 μ g of plasmid pNZ40K-C or pNZ40K-S for use in recombination, the mixture was suspended in Saline G (0.14 M sodium chloride, 0.5 mM potassium chloride, 1.1 mM disodium hydrogenphosphate, 1.5 mM potassium dihydrogenphosphate, 0.5 mM magnesium chloride
 15 hexahydrate, 0.011% glucose). The suspension was subjected to electrophoresis under conditions of 3.0 kV cm^{-1} , 0.4 msec and 25°C, using ^{GENE PULSER (trademark)} ~~Gene Pulser~~ (manufactured by Bio-Rad Co., Ltd.) at room temperature. The plasmid-infected cells were then cultured at 37°C for 72 hours.
 20 The cells were lysed by freezing and thawing 3 times to recover viruses containing the recombinant virus.

- The recovered recombinant virus was selected as follows. The recovered viral solution was infected to monolayered CEF and 10 ml of agar solution containing
 25 growth medium was overlaid thereon. After agar was warmed at room temperature, incubation was performed at 37°C until plaques of FPV appeared. Then agar medium

containing Blueo-gal in a concentration of 200 µg/ml was overlaid on the agar followed by incubation at 37°C for further 48 hours. Among all of the plaques, about 1% of the plaques were colored blue. These blue plaques were isolated and recovered. By the same procedures, isolation and recovery were repeated to purify the virus until all the plaques were stained to blue. In general, the repeated procedures were terminated by 3 to 4 days. The purified strains were named 40K-C and 40K-S, respectively. In 40K-C and 40K-S, each position of the DNAs inserted was confirmed by dot blotting hybridization and Southern blotting hybridization.

Example 4

Expression of TTM-1 polypeptide in cells infected with 40K-C and 40K-S

In order to confirm that 40K-C and 40K-S could express TTM-1 polypeptide in infected cells, Western blotting was performed using anti-Mycoplasma gallisepticum S6 strain sera. Virus 40K-C or 40K-S was infected to CEF and cultured at 37°C until plaques were formed. The cells were then scraped off with a cell scraper and centrifuged at 8000G for 20 minutes together with the culture supernatant. The cell-containing precipitates (hereinafter referred to as pellets) were recovered. After washing with PBS, the pellets were centrifuged at 8000G for 20 minutes followed by rinsing to recover the pellets. The pellets were then suspended

in 150 μ l of PBS. From the suspension 50 μ l was taken and added with the same volume of Laemmli's buffer (containing 10% mercapto-ethanol). After boiling for 3 minutes, the mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (hereinafter abbreviated as SDS-PAGE) in accordance with the Laemmli's method (Nature, 227, 668-685 (1970)). The polypeptides isolated on the SDS-PAGE-completed gel were transferred onto a polyvinylidene difluoride membrane (~~Immobilon~~ ^{IMMOBILON (trademark)} Transfer Membrane, made by Millipore Inc., hereinafter simply referred to as membrane) according to the method of Burnett et al., (A. Anal. Biochem., 112, 195-203 (1970)) or by the method of Towbin et al. (Proc. Natl. Acad. Sci., 75, 4350-4354 (1979)) by means of electrophoresis. The membrane was dipped for an hour into PBS containing 3% skimmed milk for blocking not to cause any non-specific binding. Next, the membrane was dipped for an hour in PBS in which chick anti-Mycoplasma gallisepticum S6 strain serum was diluted to 1000-fold.

Subsequently, the membrane was rinsed with PBS and then dipped for an hour in PBS containing alkaline phosphatase conjugate anti-chick IgG as a secondary antibody. After the membrane was rinsed with PBS, a color-forming reaction was carried out in 10 ml of a solution containing 100 mM Tris hydrochloride (pH 7.5), 0.15 M sodium chloride and 50 mM magnesium chloride, using Nitro Blue Tetrazolium salt (NBT, made by GIBCO-BRL Inc.) and 5-bromo-4-chloro-3-indole phosphate-p-toluidine

(BCIP, made by GIBCO-BRL Inc.) as color-forming substrates.

The results of the Western blotting are shown in Fig. 7.

5 As shown in Fig. 7, proteins could be confirmed with the cells infected both with 40K-S and 40K-C as those reactive at the objective positions. It was thus verified that the expected proteins could be expressed in the recombinant FPV infected cells.

10 Example 5

Antibody-inducing capability of recombinant FPV-inoculated chicken

After 40K-C and 40K-S were cultured in CEF at 37°C for 48 hours, the procedure of freezing and thawing
 15 was repeated twice to recover the cell suspension. The cell suspension was adjusted to have a virus titer of 10^6 pfu/ml and then inoculated to SPF chicken (Line M, Nippon Seibutsu Kagaku Kenkyusho) of 7 days old at the right wing web in a dose of 10 μ l through a stab needle. After
 20 the inoculation, take of the pock was observed and the sera were collected 2 weeks after the inoculation. The antibody titer of the sera collected was determined by
 a (Enzyme Linked Immunosorbent Assay) ELISA. The purified TTM-1 polypeptide was dissolved in a bicarbonate buffer solution in a concentration of 1
 25 μ g/well. After adsorption to a 96 well microtiter plate, blocking was effected with skimmed milk to prevent the subsequent non-specific adsorption. Next, a dilution of

the sample serum was charged in each well and then horse
 radish peroxide-labeled anti-chicken immunoglobulin
 antibody (rabbit antibody) was added thereto as a
 secondary antibody. After thoroughly washing, 2,2'-
 5 azinodiethylbenzothiazoline sulfonate was added to the
 mixture as a substrate and a relative dilution
 magnification of the antibody was measured with an
 immuno-reader in terms of absorbance at a wavelength of
 405 nm. As a primary antibody for control, anti-TTM-1
 10 polypeptide chicken serum was used. The results are
 shown in Table 1.

Table 1. Antibody titer of rFPV-inoculated
 chicken by ELISA

15	Methods for treating chicken	Antibody titer of anti- TTM-1 polypeptide
	40K-S inoculation	1024
	40K-C inoculation	512
	TTM-1 immunization	512
	non-inoculated	1

20 Antibody titer: Dilution magnification when the
 antibody titer of the group of non-
 inoculated chicken serum dilution was
 made 1

As shown in Table 1, the results reveal that
 25 when 40K-C or 40K-S was inoculated to chicken, the anti-
 TTM-1 antibody titer in sera was increased to the level

higher than the antibody titer in sera from the chicken immunized with TTM-1 polypeptide. From the results it was confirmed that the recombinant FPV could significantly induce the antibody titer to the inoculated chicken.

Example 6

Mycoplasma challenge test against recombinant FPV-inoculated chicken

The challenge test was conducted basically in accordance with the standard for biological preparations for animals. The method is briefly described below.

Strains 40K-C and 40K-S were inoculated to SPF chicken (Line M, Japan Biological Science Laboratory) of 5 weeks old at the right wing web in a dose of 10 μ l through a stab needle. After the inoculation, take of the pock was observed to verify completion of the immunization. Two weeks after the inoculation,

Mycoplasma gallisepticum strain R was forced to be intratracheally administered in a dose of 10^4 to 10^5 cfu/chick, whereby infection was made sure. On Day 14

after the infection, the chicken were euthanized with ^a NEMBTAL (trade name) (manufactured by Abbott) Numbutal. Tissue sections were prepared from the tracheal lesion and scores of the tracheal lesion were determined based on the thickness of tracheal mucous membrane and histological findings. The scores were also determined by the above standard for biological preparations. An average of scores for the tracheal

lesion observed with each chick in the groups was made the score for the respective groups. For information, criteria to determine tracheal lesion scores is shown in Table 2.

Table 2. Standard Criteria for Scoring Tracheal Lesion

Thickness of Mucous Membrane	Histological Finding	Score
90 μ m ~	normal appearance of ciliated epithelial cells and mucus gland	0
	In the lamina propria, slight infiltration of round cells or minute nest can be found, but epithelial cell-layer is normal.	1
90 μ m ~ 110 μ m	Epithelial cell are degenerated or disseminated, and the lamina propria is moderately thickened due to round cells infiltration.	2
	Squamous metaplasia of surface epithelium and lamina propria is extremely thickened due to capillary hyperplasia and rounded cells infiltration; cell debris are accumulated in the tracheal lumen.	3
110 μ m ~		

The results of evaluation are shown in Table 3 and Fig. 8.

Table 3. Means tracheal lesion scores in FPV-inoculated Chicken

Vaccination	Lesion Score	
	Average	Standard Error
40K-S	1.38	0.16
40K-C	1.89	0.13
Commercial vaccine	2.11	0.24
TTM-1 polypeptide	1.09	0.23
None	2.27	0.21

As is clearly noted from the results above, the lesion scores of chicken inoculated with 40K-C and 40K-S are obviously low as compared to that of the non-inoculated chicken, indicating that the vaccines of the present invention clearly imparted to chicken the effective infection prevention for Mycoplasma challenge. Thus, the results reveal that 40K-C and 40K-S could be effective vaccines for Mycoplasma gallisepticum.

INDUSTRIAL APPLICABILITY

According to the present invention, the fusion proteins of the polypeptides derived from antigenic proteins of Mycoplasma gallisepticum and the polypeptides
5 derived from outer membrane proteins of herpes viruses are obtained. The fusion proteins are effective as vaccines for anti-Mycoplasma infection, anti-chicken pox or anti-Marek's disease. By use of the hybrid DNAs coding for the fusion proteins, Mycoplasma gallisepticum
10 antigenic proteins can be efficiently provided on the surface of host cells. Moreover, the hybrid DNAs can secrete the antigenic proteins extracellularly to obtain Avipox viruses that can be efficiently recognized by the antigen recognizing cells in host cells. The thus
15 obtained recombinant Avipox viruses are useful as potent vaccines for anti-Mycoplasma infection.

Sequence:

ATG CAC TAT TTT AGG CGG AAT TGC ATA TTT TTC CTT ATA GTT ATT CTA	48
Met His Tyr Phe Arg Arg Asn Cys Ile Phe Phe Leu Ile Val Ile Leu	
1 5 10 15	
TAT GGT ACG AAC TCA TCT CCG AGT ACC CAA AAT GTG ACA TCA AGA GAA	96
Tyr Gly Thr Asn Ser Ser Pro Ser Thr Gln Asn Val Thr Ser Arg Glu	
20 25 30	

	GTT GTT TCG AGC GTC CAG TTG TCT GAG GAA GAG TCT ACG TTT TAT CTT	144
	Val Val Ser Ser Val Gln Leu Ser Glu Glu Glu Ser Thr Phe Tyr Leu	
	35 40 45	
	TGT CCC CCA CCA GTG GGT TCA ACC GTG ATC CGT CTA GAA TTC GGC TGT	192
5	Cys Pro Pro Pro Val Gly Ser Thr Val Ile Arg Leu Glu Phe Gly Cys	
	50 55 60	
	ATG TCT ATT ACT AAA AAA GAT GCA AAC CCA AAT AAT GGC CAA ACC CAA	240
	Met Ser Ile Thr Lys Lys Asp Ala Asn Pro Asn Asn Gly Gln Thr Gln	
	65 70 75 80	
10	TTA GAA GCA GCG CGA ATG GAG TTA ACA GAT CTA ATC AAT GCT AAA GCG	288
	Leu Glu Ala Ala Arg Met Glu Leu Thr Asp Leu Ile Asn Ala Lys Ala	
	85 90 95	
	ATG ACA TTA GCT TCA CTA CAA GAC TAT GCC AAG ATT GAA GCT AGT TTA	336
	Met Thr Leu Ala Ser Leu Gln Asp Tyr Ala Lys Ile Glu Ala Ser Leu	
15	100 105 110	
	TCA TCT GCT TAT AGT GAA GCT GAA ACA GTT AAC AAT AAC CTT AAT GCA	384
	Ser Ser Ala Tyr Ser Glu Ala Glu Thr Val Asn Asn Asn Leu Asn Ala	
	115 120 125	
	ACA TTA GAA CAA CTA AAA ATG GCT AAA ACT AAT TTA GAA TCA GCC ATC	432
20	Thr Leu Glu Gln Leu Lys Met Ala Lys Thr Asn Leu Glu Ser Ala Ile	
	130 135 140	
	AAC CAA GCT AAT ACG GAT AAA ACG ACT TTT GAT AAT GAA CAC CCA AAT	480
	Asn Gln Ala Asn Thr Asp Lys Thr Thr Phe Asp Asn Glu His Pro Asn	
	145 150 155 160	
25	TTA GTT GAA GCA TAC AAA GCA CTA AAA ACC ACT TTA GAA CAA CGT GCT	528
	Leu Val Glu Ala Tyr Lys Ala Leu Lys Thr Thr Leu Glu Gln Arg Ala	
	165 170 175	

ACT AAC CTT GAA GGT TTG TCA TCA ACT GCT TAT AAT CAA ATT CGC AAT 576
 Thr Asn Leu Glu Gly Leu Ser Ser Thr Ala Tyr Asn Gln Ile Arg Asn
 180 185 190
 AAT TTA GTG GAT CTA TAC AAT AAA GCT AGT AGT TTA ATA ACT AAA ACA 624
 5 Asn Leu Val Asp Leu Tyr Asn Lys Ala Ser Ser Leu Ile Thr Lys Thr
 195 200 205
 CTA GAT CCA CTA AAT GGG GGA ACG CTT TTA GAT TCT AAT GAG ATT ACT 672
 Leu Asp Pro Leu Asn Gly Gly Thr Leu Leu Asp Ser Asn Glu Ile Thr
 210 215 220
 10 ACA GCT AAT AAG AAT ATT AAT AAT ACG TTA TCA ACT ATT AAT GAA CAA 720
 Thr Ala Asn Lys Asn Ile Asn Asn Thr Leu Ser Thr Ile Asn Glu Gln
 225 230 235 240
 AAG ACT AAT GCT GAT GCA TTA TCT AAT AGT TTT ATT AAA AAA GTG ATT 768
 Lys Thr Asn Ala Asp Ala Leu Ser Asn Ser Phe Ile Lys Lys Val Ile
 15 245 250 255
 CAA AAT AAT GAA CAA AGT TTT GTA GGG ACT TTT ACA AAC GCT AAT GTT 816
 Gln Asn Asn Glu Gln Ser Phe Val Gly Thr Phe Thr Asn Ala Asn Val
 260 265 270
 CAA CCT TCA AAC TAC AGT TTT GTT GCT TTT AGT GCT GAT GTA ACA CCC 864
 20 Gln Pro Ser Asn Tyr Ser Phe Val Ala Phe Ser Ala Asp Val Thr Pro
 275 280 285
 GTC AAT TAT AAA TAT GCA AGA AGG ACC GTT TGG AAT GGT GAT GAA CCT 912
 Val Asn Tyr Lys Tyr Ala Arg Arg Thr Val Trp Asn Gly Asp Glu Pro
 290 295 300
 25 TCA AGT AGA ATT CTT GCA AAC ACG AAT AGT ATC ACA GAT GTT TCT TGG 960
 Ser Ser Arg Ile Leu Ala Asn Thr Asn Ser Ile Thr Asp Val Ser Trp
 305 310 315 320

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	ATT TAT AGT TTA GCT GGA ACA AAC ACG AAG TAC CAA TTT AGT TTT AGC	1008
	Ile Tyr Ser Leu Ala Gly Thr Asn Thr Lys Tyr Gln Phe Ser Phe Ser	
	325 330 335	
	AAC TAT GGT CCA TCA ACT GGT TAT TTA TAT TTC CCT TAT AAG TTG GTT	1056
5	Asn Tyr Gly Pro Ser Thr Gly Tyr Leu Tyr Phe Pro Tyr Lys Leu Val	
	340 345 350	
	AAA GCA GCT GAT GCT AAT AAC GTT GGA TTA CAA TAC AAA TTA AAT AAT	1104
	Lys Ala Ala Asp Ala Asn Asn Val Gly Leu Gln Tyr Lys Leu Asn Asn	
	355 360 365	
10	GGA AAT GTT CAA CAA GTT GAG TTT GCC ACT TCA ACT AGT GCA AAT AAT	1152
	Gly Asn Val Gln Gln Val Glu Phe Ala Thr Ser Thr Ser Ala Asn Asn	
	370 375 380	
	ACT ACA GCT AAT CCA ACT CCA GCA GTT GAT GAG ATT AAA GTT GCT AAA	1200
	Thr Thr Ala Asn Pro Thr Pro Ala Val Asp Glu Ile Lys Val Ala Lys	
15	385 390 395 400	
	ATC GTT TTA TCA GGT TTA AGA TTT GGC CAA AAC ACA ATC GAA TTA AGT	1248
	Ile Val Leu Ser Gly Leu Arg Phe Gly Gln Asn Thr Ile Glu Leu Ser	
	405 410 415	
	GTT CCA ACG GGT GAA GGA AAT ATG AAT AAA GTT GCG CCA ATG ATT GGC	1296
20	Val Pro Thr Gly Glu Gly Asn Met Asn Lys Val Ala Pro Met Ile Gly	
	420 425 430	
	AAC ATT TAT CTT AGC TCA AAT GAA AAT AAT GCT GAT AAG ATC CCC GGG	1344
	Asn Ile Tyr Leu Ser Ser Asn Glu Asn Asn Ala Asp Lys Ile Pro Gly	
	435 440 445	
25	TAC CGT CGA CCC GGT ACA TTT TTA TAA	1371
	Tyr Arg Arg Pro Gly Thr Phe Leu ***	
	450 455	

SEQUENCE LISTING

SEQ NO: 2

Length of sequence: 456

Type of sequence: amino acid

5 Topology: linear

Kind of sequence: protein

Sequence:

Met	His	Tyr	Phe	Arg	Arg	Asn	Cys	Ile	Phe	Phe	Leu	Ile	Val	Ile	Leu
1				5					10					15	
Tyr	Gly	Thr	Asn	Ser	Ser	Pro	Ser	Thr	Gln	Asn	Val	Thr	Ser	Arg	Glu
			20					25					30		
Val	Val	Ser	Ser	Val	Gln	Leu	Ser	Glu	Glu	Glu	Ser	Thr	Phe	Tyr	Leu
		35				40						45			
Cys	Pro	Pro	Pro	Val	Gly	Ser	Thr	Val	Ile	Arg	Leu	Glu	Phe	Gly	Cys
	50					55					60				
Met	Ser	Ile	Thr	Lys	Lys	Asp	Ala	Asn	Pro	Asn	Asn	Gly	Gln	Thr	Gln
65				70					75					80	
Leu	Glu	Ala	Ala	Arg	Met	Glu	Leu	Thr	Asp	Leu	Ile	Asn	Ala	Lys	Ala
				85					90					95	
Met	Thr	Leu	Ala	Ser	Leu	Gln	Asp	Tyr	Ala	Lys	Ile	Glu	Ala	Ser	Leu
		100						105					110		
Ser	Ser	Ala	Tyr	Ser	Glu	Ala	Glu	Thr	Val	Asn	Asn	Asn	Leu	Asn	Ala
		115						120					125		
Thr	Leu	Glu	Gln	Leu	Lys	Met	Ala	Lys	Thr	Asn	Leu	Glu	Ser	Ala	Ile
		130				135						140			
Asn	Gln	Ala	Asn	Thr	Asp	Lys	Thr	Thr	Phe	Asp	Asn	Glu	His	Pro	Asn
145						150					155				160

Leu Val Glu Ala Tyr Lys Ala Leu Lys Thr Thr Leu Glu Gln Arg Ala
 165 170 175
 Thr Asn Leu Glu Gly Leu Ser Ser Thr Ala Tyr Asn Gln Ile Arg Asn
 180 185 190
 Asn Leu Val Asp Leu Tyr Asn Lys Ala Ser Ser Leu Ile Thr Lys Thr
 195 200 205
 Leu Asp Pro Leu Asn Gly Gly Thr Leu Leu Asp Ser Asn Glu Ile Thr
 210 215 220
 Thr Ala Asn Lys Asn Ile Asn Asn Thr Leu Ser Thr Ile Asn Glu Gln
 225 230 235 240
 Lys Thr Asn Ala Asp Ala Leu Ser Asn Ser Phe Ile Lys Lys Val Ile
 245 250 255
 Gln Asn Asn Glu Gln Ser Phe Val Gly Thr Phe Thr Asn Ala Asn Val
 260 265 270
 Gln Pro Ser Asn Tyr Ser Phe Val Ala Phe Ser Ala Asp Val Thr Pro
 275 280 285
 Val Asn Tyr Lys Tyr Ala Arg Arg Thr Val Trp Asn Gly Asp Glu Pro
 290 295 300
 Ser Ser Arg Ile Leu Ala Asn Thr Asn Ser Ile Thr Asp Val Ser Trp
 305 310 315 320
 Ile Tyr Ser Leu Ala Gly Thr Asn Thr Lys Tyr Gln Phe Ser Phe Ser
 325 330 335
 Asn Tyr Gly Pro Ser Thr Gly Tyr Leu Tyr Phe Pro Tyr Lys Leu Val
 340 345 350
 Lys Ala Ala Asp Ala Asn Asn Val Gly Leu Gln Tyr Lys Leu Asn Asn
 355 360 365
 Gly Asn Val Gln Gln Val Glu Phe Ala Thr Ser Thr Ser Ala Asn Asn
 370 375 380

SEQUENCE LISTING

SEQ NO: 3

Length of sequence: 3261

Type of sequence: nucleic acid

5 Number of strand: double strand

Topology: linear

Kind of sequence: other nucleic acid, hybrid DNA (40K-C)

Sequence:

ATG CAC TAT TTT AGG CGG AAT TGC ATA TTT TTC CTT ATA GTT ATT CTA	48
Met His Tyr Phe Arg Arg Asn Cys Ile Phe Phe Leu Ile Val Ile Leu	
1 5 10 15	
TAT GGT ACG AAC TCA TCT CCG AGT ACC CAA AAT GTG ACA TCA AGA GAA	96
Tyr Gly Thr Asn Ser Ser Pro Ser Thr Gln Asn Val Thr Ser Arg Glu	
20 25 30	
GTT GTT TCG AGC GTC CAG TTG TCT GAG GAA GAG TCT ACG TTT TAT CTT	144
Val Val Ser Ser Val Gln Leu Ser Glu Glu Glu Ser Thr Phe Tyr Leu	
35 40 45	

TGT CCC CCA CCA GTG GGT TCA ACC GTG ATC CGT CTA GAA CCG CCG CGA 192
 Cys Pro Pro Pro Val Gly Ser Thr Val Ile Arg Leu Glu Pro Pro Arg
 50 55 60
 AAA TGT CCC GAA CCT AGA AAA GCC ACC GAG TGG GGT GAA GGA ATC GCG 240
 Lys Cys Pro Glu Pro Arg Lys Ala Thr Glu Trp Gly Glu Gly Ile Ala
 65 70 75 80
 ATA TTA TTT AAA GAG AAT ATC AGT CCA TAT AAA TTT AAA GTG ACG CTT 288
 Ile Leu Phe Lys Glu Asn Ile Ser Pro Tyr Lys Phe Lys Val Thr Leu
 85 90 95
 TAT TAT AAA AAT ATC ATT CAG ACG ACG ACA TGG ACG GGG ACG ACA TAT 336
 Tyr Tyr Lys Asn Ile Ile Gln Thr Thr Thr Trp Thr Gly Thr Thr Tyr
 100 105 110
 AGA CAG ATC ACT AAT CGA TAT ACA GAT AGG ACG CCC GTT TCC ATT GAA 384
 Arg Gln Ile Thr Asn Arg Tyr Thr Asp Arg Thr Pro Val Ser Ile Glu
 115 120 125
 GAG ATC ACG GAT CTA ATC GAC GGC AAA GGA AGA TGC TCA TCT AAA GCA 432
 Glu Ile Thr Asp Leu Ile Asp Gly Lys Gly Arg Cys Ser Ser Lys Ala
 130 135 140
 AGA TAC CTT AGA AAC AAT GTA TAT GTT GAA GCG TTT GAC AGG GAT GCG 480
 Arg Tyr Leu Arg Asn Asn Val Tyr Val Glu Ala Phe Asp Arg Asp Ala
 145 150 155 160
 GGA GAA AAA CAA GTA CTT CTA AAA CCA TCA AAA TTC AAC ACG CCC GAA 528
 Gly Glu Lys Gln Val Leu Leu Lys Pro Ser Lys Phe Asn Thr Pro Glu
 165 170 175
 TCT AGG GCA TGG CAC ACG ACT AAT GAG ACG TAT ACC GTG TGG GGA TCA 576
 Ser Arg Ala Trp His Thr Thr Asn Glu Thr Tyr Thr Val Trp Gly Ser
 180 185 190

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CCA TGG ATA TAT CGA ACG GGA ACC TCC GTC AAT TGT ATA GTA GAG GAA	624
Pro Trp Ile Tyr Arg Thr Gly Thr Ser Val Asn Cys Ile Val Glu Glu	
195 200 205	
ATG GAT GCC CGC TCT GTG TTT CCG TAT TCA TAT TTT GCA ATG GCC AAT	672
Met Asp Ala Arg Ser Val Phe Pro Tyr Ser Tyr Phe Ala Met Ala Asn	
210 215 220	
GGC GAC ATC GCG AAC ATA TCT CCA TTT TAT GGT CTA TCC CCA CCA GAG	720
Gly Asp Ile Ala Asn Ile Ser Pro Phe Tyr Gly Leu Ser Pro Pro Glu	
225 230 235 240	
GCT GCC GCA GAA CCC ATG GGA TAT CCC CAG GAT AAT TTC AAA CAA CTA	768
Ala Ala Ala Glu Pro Met Gly Tyr Pro Gln Asp Asn Phe Lys Gln Leu	
245 250 255	
GAT AGC TAT TTT TCA ATG GAT TTG GAC AAG CGT CGA AAA GCA AGC CTT	816
Asp Ser Tyr Phe Ser Met Asp Leu Asp Lys Arg Arg Lys Ala Ser Leu	
260 265 270	
CCA GTC AAG CGT AAC TTT CTC ATC ACA TCA CAC TTC ACA GTT GGG TGG	864
Pro Val Lys Arg Asn Phe Leu Ile Thr Ser His Phe Thr Val Gly Trp	
275 280 285	
GAC TGG GCT CCA AAA ACT ACT CGT GTA TGT TCA ATG ACT AAG TGG AAA	912
Asp Trp Ala Pro Lys Thr Thr Arg Val Cys Ser Met Thr Lys Trp Lys	
290 295 300	
GAG GTG ACT GAA ATG TTG CGT GCA ACA GTT AAT GGG AGA TAC AGA TTT	960
Glu Val Thr Glu Met Leu Arg Ala Thr Val Asn Gly Arg Tyr Arg Phe	
305 310 315 320	
ATG GCC CGT GAA CTT TCG GCA ACG TTT ATC AGT AAT ACG ACT GAG TTT	1008
Met Ala Arg Glu Leu Ser Ala Thr Phe Ile Ser Asn Thr Thr Glu Phe	
325 330 335	

GAT CCA AAT CGC ATC ATA TTA GGA CAA TGT ATT AAA CGC GAG GCA GAA 1056
 Asp Pro Asn Arg Ile Ile Leu Gly Gln Cys Ile Lys Arg Glu Ala Glu
 340 345 350
 GCA GCA ATC GAG CAG ATA TTT AGG ACA AAA TAT AAT GAC AGT CAC GTC 1104
 Ala Ala Ile Glu Gln Ile Phe Arg Thr Lys Tyr Asn Asp Ser His Val
 355 360 365
 AAG GTT GGA CAT GTA CAA TAT TTC TTG GCT CTC GGG GGA TTT ATT GTA 1152
 Lys Val Gly His Val Gln Tyr Phe Leu Ala Leu Gly Gly Phe Ile Val
 370 375 380
 GCA TAT CAG CCT GTT CTA TCC AAA TCC CTG GCT CAT ATG TAC CTC AGA 1200
 Ala Tyr Gln Pro Val Leu Ser Lys Ser Leu Ala His Met Tyr Leu Arg
 385 390 395 400
 GAA TTG ATG AGA GAC AAC AGG ACC GAT GAG ATG CTC GAC CTG GTA AAC 1248
 Glu Leu Met Arg Asp Asn Arg Thr Asp Glu Met Leu Asp Leu Val Asn
 405 410 415
 AAT AAG CAT GCA ATT TAT AAG AAA AAT GCT ACC TCA TTG TCA CGA TTG 1296
 Asn Lys His Ala Ile Tyr Lys Lys Asn Ala Thr Ser Leu Ser Arg Leu
 420 425 430
 CGG CGA GAT ATT CGA AAT GCA CCA AAT AGA AAA ATA ACA TTA GAC GAC 1344
 Arg Arg Asp Ile Arg Asn Ala Pro Asn Arg Lys Ile Thr Leu Asp Asp
 435 440 445
 ACC ACA GCT ATT AAA TCG ACA TCG TCT GTT CAA TTC GCC ATG CTC CAA 1392
 Thr Thr Ala Ile Lys Ser Thr Ser Ser Val Gln Phe Ala Met Leu Gln
 450 455 460
 TTT CTT TAT GAT CAT ATA CAA ACC CAT ATT AAT GAT ATG TTT AGT AGG 1440
 Phe Leu Tyr Asp His Ile Gln Thr His Ile Asn Asp Met Phe Ser Arg
 465 470 475 480

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ATT GCC ACA GCT TGG TGC GAA TTG CAG AAT AGA GAA CTT GTT TTA TGG 1488
 Ile Ala Thr Ala Trp Cys Glu Leu Gln Asn Arg Glu Leu Val Leu Trp
 485 490 495
 CAC GAA GGG ATA AAG ATT AAT CCT AGC GCT ACA GCG AGT GCA ACA TTA 1536
 His Glu Gly Ile Lys Ile Asn Pro Ser Ala Thr Ala Ser Ala Thr Leu
 500 505 510
 GGA AGG AGA GTG GCT GCA AAG ATG TTG GGG GAT GTC GCT GCT GTA TCG 1584
 Gly Arg Arg Val Ala Ala Lys Met Leu Gly Asp Val Ala Ala Val Ser
 515 520 525
 AGC TGC ACT GCT ATA GAT GCG GAA TCC GTC ACT TTG CAA AAT TCT ATG 1632
 Ser Cys Thr Ala Ile Asp Ala Glu Ser Val Thr Leu Gln Asn Ser Met
 530 535 540
 CGA GTT ATC ACA TCC ACT AAT ACA TGT TAT AGC CGA CCA TTG GTT CTA 1680
 Arg Val Ile Thr Ser Thr Asn Thr Cys Tyr Ser Arg Pro Leu Val Leu
 545 550 555 560
 TTT TCA TAT GGA GAA AAC CAA GGA AAC ATA CAG GGA CAA CTC GGT GAA 1728
 Phe Ser Tyr Gly Glu Asn Gln Gly Asn Ile Gln Gly Gln Leu Gly Glu
 565 570 575
 AAC AAC GAG TTG CTT CCA ACG CTA GAG GCT GTA GAG CCA TGC TCG GCT 1776
 Asn Asn Glu Leu Leu Pro Thr Leu Glu Ala Val Glu Pro Cys Ser Ala
 580 585 590
 AAT CAT CGT AGA TAT TTT CTG TTT GGA TCC GGT TAT GCT TTA TTT GAA 1824
 Asn His Arg Arg Tyr Phe Leu Phe Gly Ser Gly Tyr Ala Leu Phe Glu
 595 600 605
 AAC TAT AAT TTT GTT AAG ATG GTA GAC GCT GCC GAT ATA CAG ATT GCT 1872
 Asn Tyr Asn Phe Val Lys Met Val Asp Ala Ala Asp Ile Gln Ile Ala
 610 615 620

AAT TTA GAA TCA GCC ATC AAC CAA GCT AAT ACG GAT AAA ACG ACT TTT 2352
 Asn Leu Glu Ser Ala Ile Asn Gln Ala Asn Thr Asp Lys Thr Thr Phe
 770 775 780
 GAT AAT GAA CAC CCA AAT TTA GTT GAA GCA TAC AAA GCA CTA AAA ACC 2400
 Asp Asn Glu His Pro Asn Leu Val Glu Ala Tyr Lys Ala Leu Lys Thr
 785 790 795 800
 ACT TTA GAA CAA CGT GCT ACT AAC CTT GAA GGT TTG TCA TCA ACT GCT 2448
 Thr Leu Glu Gln Arg Ala Thr Asn Leu Glu Gly Leu Ser Ser Thr Ala
 805 810 815
 TAT AAT CAA ATT CGC AAT AAT TTA GTG GAT CTA TAC AAT AAA GCT AGT 2496
 Tyr Asn Gln Ile Arg Asn Asn Leu Val Asp Leu Tyr Asn Lys Ala Ser
 820 825 830
 AGT TTA ATA ACT AAA ACA CTA GAT CCA CTA AAT GGG GGA ACG CTT TTA 2544
 Ser Leu Ile Thr Lys Thr Leu Asp Pro Leu Asn Gly Gly Thr Leu Leu
 835 840 845
 GAT TCT AAT GAG ATT ACT ACA GCT AAT AAG AAT ATT AAT AAT ACG TTA 2592
 Asp Ser Asn Glu Ile Thr Thr Ala Asn Lys Asn Ile Asn Asn Thr Leu
 850 855 860
 TCA ACT ATT AAT GAA CAA AAG ACT AAT GCT GAT GCA TTA TCT AAT AGT 2640
 Ser Thr Ile Asn Glu Gln Lys Thr Asn Ala Asp Ala Leu Ser Asn Ser
 865 870 875 880
 TTT ATT AAA AAA GTG ATT CAA AAT AAT GAA CAA AGT TTT GTA GGG ACT 2688
 Phe Ile Lys Lys Val Ile Gln Asn Asn Glu Gln Ser Phe Val Gly Thr
 885 890 895
 TTT ACA AAC GCT AAT GTT CAA CCT TCA AAC TAC AGT TTT GTT GCT TTT 2736
 Phe Thr Asn Ala Asn Val Gln Pro Ser Asn Tyr Ser Phe Val Ala Phe
 900 905 910

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AGT GCT GAT GTA ACA CCC GTC AAT TAT AAA TAT GCA AGA AGG ACC GTT	2784
Ser Ala Asp Val Thr Pro Val Asn Tyr Lys Tyr Ala Arg Arg Thr Val	
915 920 925	
TGG AAT GGT GAT GAA CCT TCA AGT AGA ATT CTT GCA AAC ACG AAT AGT	2832
Trp Asn Gly Asp Glu Pro Ser Ser Arg Ile Leu Ala Asn Thr Asn Ser	
930 935 940	
ATC ACA GAT GTT TCT TGG ATT TAT AGT TTA GCT GGA ACA AAC ACG AAG	2880
Ile Thr Asp Val Ser Trp Ile Tyr Ser Leu Ala Gly Thr Asn Thr Lys	
945 950 955 960	
TAC CAA TTT AGT TTT AGC AAC TAT GGT CCA TCA ACT GGT TAT TTA TAT	2928
Tyr Gln Phe Ser Phe Ser Asn Tyr Gly Pro Ser Thr Gly Tyr Leu Tyr	
965 970 975	
TTC CCT TAT AAG TTG GTT AAA GCA GCT GAT GCT AAT AAC GTT GGA TTA	2976
Phe Pro Tyr Lys Leu Val Lys Ala Ala Asp Ala Asn Asn Val Gly Leu	
980 985 990	
CAA TAC AAA TTA AAT AAT GGA AAT GTT CAA CAA GTT GAG TTT GCC ACT	3024
Gln Tyr Lys Leu Asn Asn Gly Asn Val Gln Gln Val Glu Phe Ala Thr	
995 1000 1005	
TCA ACT AGT GCA AAT AAT ACT ACA GCT AAT CCA ACT CCA GCA GTT GAT	3072
Ser Thr Ser Ala Asn Asn Thr Thr Ala Asn Pro Thr Pro Ala Val Asp	
1010 1015 1020	
GAG ATT AAA GTT GCT AAA ATC GTT TTA TCA GGT TTA AGA TTT GGC CAA	3120
Glu Ile Lys Val Ala Lys Ile Val Leu Ser Gly Leu Arg Phe Gly Gln	
1025 1030 1035 1040	
AAC ACA ATC GAA TTA AGT GTT CCA ACG GGT GAA GGA AAT ATG AAT AAA	3168
Asn Thr Ile Glu Leu Ser Val Pro Thr Gly Glu Gly Asn Met Asn Lys	
1045 1050 1055	

GTT GCG CCA ATG ATT GGC AAC ATT TAT CTT AGC TCA AAT GAA AAT AAT 3216
Val Ala Pro Met Ile Gly Asn Ile Tyr Leu Ser Ser Asn Glu Asn Asn

1060

1065

1070

GCT GAT AAG ATC CCC GGG TAC CGT CGA CCC GGT ACA TTT TTA TAA 3261
Ala Asp Lys Ile Pro Gly Tyr Arg Arg Pro Gly Thr Phe Leu ***

1075

1080

1085

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SEQUENCE LISTING

SEQ NO: 4

Length of sequence: 1086

Type of sequence: amino acid

5 Topology: linear

Kind of sequence: protein

Sequence:

Met	His	Tyr	Phe	Arg	Arg	Asn	Cys	Ile	Phe	Phe	Leu	Ile	Val	Ile	Leu
1					5					10				15	
Tyr	Gly	Thr	Asn	Ser	Ser	Pro	Ser	Thr	Gln	Asn	Val	Thr	Ser	Arg	Glu
			20					25					30		
Val	Val	Ser	Ser	Val	Gln	Leu	Ser	Glu	Glu	Glu	Ser	Thr	Phe	Tyr	Leu
		35				40						45			
Cys	Pro	Pro	Pro	Val	Gly	Ser	Thr	Val	Ile	Arg	Leu	Glu	Pro	Pro	Arg
	50					55					60				
Lys	Cys	Pro	Glu	Pro	Arg	Lys	Ala	Thr	Glu	Trp	Gly	Glu	Gly	Ile	Ala
65					70					75				80	
Ile	Leu	Phe	Lys	Glu	Asn	Ile	Ser	Pro	Tyr	Lys	Phe	Lys	Val	Thr	Leu
			85					90					95		
Tyr	Tyr	Lys	Asn	Ile	Ile	Gln	Thr	Thr	Thr	Trp	Thr	Gly	Thr	Thr	Tyr
			100					105					110		

Arg Gln Ile Thr Asn Arg Tyr Thr Asp Arg Thr Pro Val Ser Ile Glu
 115 120 125
 Glu Ile Thr Asp Leu Ile Asp Gly Lys Gly Arg Cys Ser Ser Lys Ala
 130 135 140
 Arg Tyr Leu Arg Asn Asn Val Tyr Val Glu Ala Phe Asp Arg Asp Ala
 145 150 155 160
 Gly Glu Lys Gln Val Leu Leu Lys Pro Ser Lys Phe Asn Thr Pro Glu
 165 170 175
 Ser Arg Ala Trp His Thr Thr Asn Glu Thr Tyr Thr Val Trp Gly Ser
 180 185 190
 Pro Trp Ile Tyr Arg Thr Gly Thr Ser Val Asn Cys Ile Val Glu Glu
 195 200 205
 Met Asp Ala Arg Ser Val Phe Pro Tyr Ser Tyr Phe Ala Met Ala Asn
 210 215 220
 Gly Asp Ile Ala Asn Ile Ser Pro Phe Tyr Gly Leu Ser Pro Pro Glu
 225 230 235 240
 Ala Ala Ala Glu Pro Met Gly Tyr Pro Gln Asp Asn Phe Lys Gln Leu
 245 250 255
 Asp Ser Tyr Phe Ser Met Asp Leu Asp Lys Arg Arg Lys Ala Ser Leu
 260 265 270
 Pro Val Lys Arg Asn Phe Leu Ile Thr Ser His Phe Thr Val Gly Trp
 275 280 285
 Asp Trp Ala Pro Lys Thr Thr Arg Val Cys Ser Met Thr Lys Trp Lys
 290 295 300
 Glu Val Thr Glu Met Leu Arg Ala Thr Val Asn Gly Arg Tyr Arg Phe
 305 310 315 320
 Met Ala Arg Glu Leu Ser Ala Thr Phe Ile Ser Asn Thr Thr Glu Phe
 325 330 335

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Asp Pro Asn Arg Ile Ile Leu Gly Gln Cys Ile Lys Arg Glu Ala Glu
 340 345 350
 Ala Ala Ile Glu Gln Ile Phe Arg Thr Lys Tyr Asn Asp Ser His Val
 355 360 365
 Lys Val Gly His Val Gln Tyr Phe Leu Ala Leu Gly Gly Phe Ile Val
 370 375 380
 Ala Tyr Gln Pro Val Leu Ser Lys Ser Leu Ala His Met Tyr Leu Arg
 385 390 395 400
 Glu Leu Met Arg Asp Asn Arg Thr Asp Glu Met Leu Asp Leu Val Asn
 405 410 415
 Asn Lys His Ala Ile Tyr Lys Lys Asn Ala Thr Ser Leu Ser Arg Leu
 420 425 430
 Arg Arg Asp Ile Arg Asn Ala Pro Asn Arg Lys Ile Thr Leu Asp Asp
 435 440 445
 Thr Thr Ala Ile Lys Ser Thr Ser Ser Val Gln Phe Ala Met Leu Gln
 450 455 460
 Phe Leu Tyr Asp His Ile Gln Thr His Ile Asn Asp Met Phe Ser Arg
 465 470 475 480
 Ile Ala Thr Ala Trp Cys Glu Leu Gln Asn Arg Glu Leu Val Leu Trp
 485 490 495
 His Glu Gly Ile Lys Ile Asn Pro Ser Ala Thr Ala Ser Ala Thr Leu
 500 505 510
 Gly Arg Arg Val Ala Ala Lys Met Leu Gly Asp Val Ala Ala Val Ser
 515 520 525
 Ser Cys Thr Ala Ile Asp Ala Glu Ser Val Thr Leu Gln Asn Ser Met
 530 535 540
 Arg Val Ile Thr Ser Thr Asn Thr Cys Tyr Ser Arg Pro Leu Val Leu
 545 550 555 560

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Phe Ser Tyr Gly Glu Asn Gln Gly Asn Ile Gln Gly Gln Leu Gly Glu
 565 570 575
 Asn Asn Glu Leu Leu Pro Thr Leu Glu Ala Val Glu Pro Cys Ser Ala
 580 585 590
 Asn His Arg Arg Tyr Phe Leu Phe Gly Ser Gly Tyr Ala Leu Phe Glu
 595 600 605
 Asn Tyr Asn Phe Val Lys Met Val Asp Ala Ala Asp Ile Gln Ile Ala
 610 615 620
 Ser Thr Phe Val Glu Leu Asn Leu Thr Leu Leu Glu Asp Arg Glu Ile
 625 630 635 640
 Leu Pro Leu Ser Val Tyr Thr Lys Glu Glu Leu Arg Asp Val Gly Val
 645 650 655
 Leu Asp Tyr Ala Glu Val Ala Arg Arg Asn Gln Leu His Glu Leu Lys
 660 665 670
 Phe Tyr Asp Ile Asn Lys Val Ile Glu Val Asp Thr Asn Tyr Ala Gly
 675 680 685
 Leu Gln Glu Phe Gly Cys Met Ser Ile Thr Lys Lys Asp Ala Asn Pro
 690 695 700
 Asn Asn Gly Gln Thr Gln Leu Glu Ala Ala Arg Met Glu Leu Thr Asp
 705 710 715 720
 Leu Ile Asn Ala Lys Ala Met Thr Leu Ala Ser Leu Gln Asp Tyr Ala
 725 730 735
 Lys Ile Glu Ala Ser Leu Ser Ser Ala Tyr Ser Glu Ala Glu Thr Val
 740 745 750
 Asn Asn Asn Leu Asn Ala Thr Leu Glu Gln Leu Lys Met Ala Lys Thr
 755 760 765
 Asn Leu Glu Ser Ala Ile Asn Gln Ala Asn Thr Asp Lys Thr Thr Phe
 770 775 780

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Asp Asn Glu His Pro Asn Leu Val Glu Ala Tyr Lys Ala Leu Lys Thr			
785	790	795	800
Thr Leu Glu Gln Arg Ala Thr Asn Leu Glu Gly Leu Ser Ser Thr Ala			
805	810	815	
Tyr Asn Gln Ile Arg Asn Asn Leu Val Asp Leu Tyr Asn Lys Ala Ser			
820	825	830	
Ser Leu Ile Thr Lys Thr Leu Asp Pro Leu Asn Gly Gly Thr Leu Leu			
835	840	845	
Asp Ser Asn Glu Ile Thr Thr Ala Asn Lys Asn Ile Asn Asn Thr Leu			
850	855	860	
Ser Thr Ile Asn Glu Gln Lys Thr Asn Ala Asp Ala Leu Ser Asn Ser			
865	870	875	880
Phe Ile Lys Lys Val Ile Gln Asn Asn Glu Gln Ser Phe Val Gly Thr			
885	890	895	
Phe Thr Asn Ala Asn Val Gln Pro Ser Asn Tyr Ser Phe Val Ala Phe			
900	905	910	
Ser Ala Asp Val Thr Pro Val Asn Tyr Lys Tyr Ala Arg Arg Thr Val			
915	920	925	
Trp Asn Gly Asp Glu Pro Ser Ser Arg Ile Leu Ala Asn Thr Asn Ser			
930	935	940	
Ile Thr Asp Val Ser Trp Ile Tyr Ser Leu Ala Gly Thr Asn Thr Lys			
945	950	955	960
Tyr Gln Phe Ser Phe Ser Asn Tyr Gly Pro Ser Thr Gly Tyr Leu Tyr			
965	970	975	
Phe Pro Tyr Lys Leu Val Lys Ala Ala Asp Ala Asn Asn Val Gly Leu			
980	985	990	
Gln Tyr Lys Leu Asn Asn Gly Asn Val Gln Gln Val Glu Phe Ala Thr			
995	1000	1005	

Ser Thr Ser Ala Asn Asn Thr Thr Ala Asn Pro Thr Pro Ala Val Asp
 1010 1015 1020
 Glu Ile Lys Val Ala Lys Ile Val Leu Ser Gly Leu Arg Phe Gly Gln
 1025 1030 1035 1040
 Asn Thr Ile Glu Leu Ser Val Pro Thr Gly Glu Gly Asn Met Asn Lys
 1045 1050 1055
 Val Ala Pro Met Ile Gly Asn Ile Tyr Leu Ser Ser Asn Glu Asn Asn
 1060 1065 1070
 Ala Asp Lys Ile Pro Gly Tyr Arg Arg Pro Gly Thr Phe Leu ***
 1075 1080 1085

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